

Histidine (His83) is Essential for Heat Shock Factor 1 (HSF1) Activation in Protecting against Acid pH Stress

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The activation of heat shock factor 1 (HSF1) can be induced by the changes in environmental pH, but the mechanism of HSF1 activation by acidification is not completely understood. This paper reports that a low pH (pH~6.0) can trigger human HSF1 activation. Considering the involvement of the imidazole group of histidine residues under acid pH stress, an *in vitro* EMSA experiment, Trp-fluorescence spectroscopy, and protein structural analysis showed that the residue, His83, is the essential for pH-dependent human HSF1-activation. To determine the roles of His83 in the HSF1-mediated stress response affecting the cellular acid resistance, mouse embryo fibroblasts with normal wild-type or mutant mouse HSF1 expression were preconditioned by heating or pH stress. The results suggest that His83 is essential for HSF1 activation or the HSF1-mediated transcription of heat shock proteins, in protecting cells from acid pH stress.

Key Words : Heat shock factor 1, Acid pH, Histidine, EMSA, Tryptophan-fluorescence spectroscopy

Introduction

Cancer cells survive and flourish within tumor tissues by exposing of an oxygen-deficient (hypoxia) and acidic extra-environment.¹ Under limited oxygen conditions, cellular glucose undergoes fermentation to lactic acid, which causes a decrease in pH within the microenvironment of a solid tumor from a normal pH of 7.3 to 7, and later to 6.5.^{2,3} In more advanced stages of cancer and metastases the extracellular pH decreases to 6.0 and to 5.7 or lower.^{4,5} Many experimental studies have reported that in most human tumors, particularly those of an epithelial origin or gliomas, the molecular chaperones, HSP70 (member of the HSP70 superfamily), HSP40 family, and HSP27 (member of the small HSP family), have been reported to show elevated mRNA and protein expression levels, revealing a potential role of heat shock factor 1 (HSF1) in carcinogenesis.⁶ In a similar vein, heat shock (elevated temperature)-induced stress was also reported to cause a significant decrease in pH (the intracellular pH decreases to 6.0) in animal cells, along with the activation of HSF1 and over-expression of HSPs.⁷ These results arouse our interest into the mechanism of HSF1 activation by acidification.

HSF1 is a central regulator of the synthesis of inducible heat shock proteins (HSPs) in mammals. In the normal state, HSF1 exists in the cytoplasm in an inert monomeric state. On the other hand, when the cells are stressed, HSF1 forms a homo-trimeric complex and is translocated into the nucleus, where it binds specifically to a conserved regulatory DNA sequence of the heat shock element (HSE), which is comprised of at least three contiguous inverted repeats of 5-nGAAn-3 upstream of the heat shock genes.^{4,5} This study examined whether a low pH in the physiological range can trigger HSF1 activation and attempted to find the key amino acids

in pH-dependent HSF1-activation.

Experimental

Plasmids and PCR-Mediated Mutagenesis. The expression vector, pET21b-human HSF1 (hHSF1) containing full-length hHSF1 cDNA, were constructed previously. The histidine residue 83 of hHSF1 was replaced with alanine residue by PCR-mediated site-directed mutagenesis.⁸ The oligonucleotides for the mutation were as follows: forward, 5'-CGGAAAGTGGTCGCCATCGAGCAGGGC-3' and reverse, 5'-GCCCTGCTCGATGGCGACCACTTCCG-3'. All plasmids were isolated from Escherichia coli JM109 and confirmed by DNA sequencing. The mouse HSF1 cDNA vector pCDNA3.1/hgy-mouse HSF1 was kindly provided by Dr. Sang-Gun Ahn, Chosun University, Korea. The special amino acids (His83 to alanine) were changed with the primers (forward, 5'-CGAAAAGTAGTCGCCATTGAGCAGGGT-3' and reverse, 5'-ACCCTGCTCAATGGCGACTACTTTCG-3').

Protein Preparation and EMSA: *E. coli* BL21 (DE3) was transformed with pET21b-human HSF1 or its histidine mutants. A point mutation of HSF1 was performed with the PCR-mediated, site-directed mutagenesis. The *E. coli* cells were cultured in LB medium to an OD₆₀₀ of 0.6-0.8, and the targeted proteins were induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and culturing for 12 h at 20 °C. The cells were then collected by centrifugation and resuspended in a binding buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM sodium chloride, and 0.5 mM PMSF. After sonication, the crude lysate was centrifuged at 13000 rpm for 40 min at 4 °C. The supernatant was loaded onto a HisTrap HP column by FPLC (GE Healthcare), and HSF1 was eluted using an elution buffer containing 250 mM

imidazole. HSF1 was further purified using a mono Q HR 5/5 column (GE Healthcare), as described previously.² The fractions that were at least 90% pure as determined by Coomassie staining were collected and stored at -80 °C. For EMSA, two complementary single-stranded HSE oligonucleotides were annealed and used in EMSA. HSE was labeled with [γ -³²P] ATP using T4 polynucleotide kinase. The dialyzed samples were incubated further with ³²P-labeled HSE [\sim 30,000 cpm (< 1 ng)] for 20 min at room temperature (25 °C). Finally, the hHSF1-HSE complexes were separated from the free HSE on a 5% Tris-borate-EDTA native gel and visualized by autoradiography.

Trp-Fluorescence Spectroscopy (TFS): The TFS samples (200 μ g/mL) were dialyzed in a TGE buffer. TFS was performed using a FP-750 spectrometer (JASCO). A wavelength of 280 nm was used for the selective excitation of tryptophan amino acid, (parameters, $\lambda_{\text{excitation}} = 280 \text{ nm}$; range of sample fluorescence emission, 300 nm-400 nm).⁹

Cell lines, Cell Culture and Transfection: Mouse embryonic fibroblast (MEF) cells (wild type and mutant *hsf1*^{-/-}) were a generous gift from Dr. Benjamin (Dr. Benjamin's Cardiology Research Lab University of Utah). The cells were cultured at 37 °C in a CO₂ incubator. The DMEM medium supplemented with 4.5 g/L glucose, 4 mM L-glutamine, penicillin, 100 μ g/ml streptomycin and 10%FBS was used as the growth medium. The mutant mouse HSF1 (pCDNA3.1-mHSF1) was transfected into the MEF *hsf1*^{-/-}, as described by Chang et al.¹⁰

Western Blot Analysis: For western blot analysis, the cells were washed twice with PBS and collected with NP-40 lysis buffer. The cell extracts were quantified using a BCA protein assay kit (Bio-Rad). Equal aliquots of the cell extracts were separated on SDS-PAGE. The proteins were then transferred to PVDF membranes, blocked and probed with the antibody against HSP70. The antibody-bound proteins were detected using an enhanced chemiluminescence substrate system (Promega).

MTT Assay: Twenty four hours after stress induction, the cell viability was evaluated using a MTT based Cell Growth Determination Kit (sigma). A MTT solution in amounts equal to 10% of the culture volume was added to the cultures. The dishes were returned to the incubator and incubated for 4 h. After incubation, the culture fluid was removed and the MTT solvent was added in amounts equal to the original culture volume. The MTT assay was carried out using the standard protocol, and the optical density was measured at 570 nm using a spectrometer.

Cell Cycle Analysis: The cells were seeded on a 10 cm plate and treated with heat or pH stress. After further incubation for 24 hours, the cells were trypsinized, pelleted and washed with PBS. The cells were fixed with 70% ethanol. After overnight incubation, the samples were washed with cold PBS and re-suspended in a DNA staining solution (20 μ g/mL propidium iodide, and 0.5 mg/mL RNase in PBS). The cells in the different phases of the cell cycle were analyzed by FACScan flow cytometry (Beckman Coulter, Cytomics FC500).

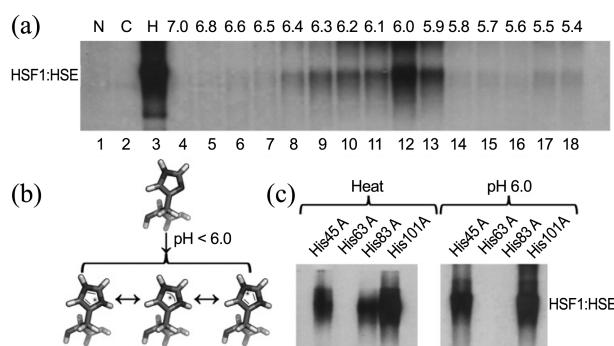


Figure 1. (a) The heat- or pH- induced DNA-binding activities of human HSF1 in the various pH buffers. The protein samples were treated with heat or various pH buffers, and then mixed with heat shock element (HSE). The hHSF1-HSE complexes were analyzed by 5% native electrophoresis gel and visualized by autoradiography. 'N' means no protein; 'C' means normal condition; 'H' means heat. (b) Protonated histidine side chain within the physiological-range pH decrease. Histidine includes the ionizable imidazole ring with an experimental pK_a value of 6.0. The acid form is a resonance hybrid of two practically equivalent contributing forms. (c) The heat- or pH- induced DNA-binding activities of human HSF1 and its histidine mutants.

Results and Discussion

This study first verified whether a low pH in the physiological range could trigger HSF1 activation, such as DNA-binding *in vitro*. Therefore, the recombinant human HSF1 protein was expressed, purified and their DNA binding activities were examined by EMSA (electrophoretic mobility shift assay) at various pH.⁴ In Figure 1, hHSF1 is inactive under non-stress conditions, which can be activated to show strong DNA-binding activity by heat (lanes 1-3 in Figure 1(a)).¹¹ Moreover, the low-pH (physiological range) environment ($5.8 < \text{pH} < 6.4$), which involve increasing amounts of hydrogen ions, gradually increased the formation of heat-induced HSF1-HSE complexes (Figure 1(a)). On the other hand, when treated with a much higher concentration of H⁺, HSF1 had inhibitory effects on the process of DNA-binding (lanes 14-18 in Figure 1(a)). These results strongly suggest that HSE binding of hHSF1 were regulated by the H⁺ concentration.¹²

The pH-dependent the protein activity is regulated preferably by the pK_a of the amino acid side chains. Of the side chains in the 20 known amino acids, the imidazole ring of a single histidine molecule has an experimental pK_a of 6.0. This means that the histidine imidazole group is the only amino acid in the proteins to be affected significantly by a physiological-ranged decrease in pH¹³ (Figure 1(b)). After protonation, the imidazole ring shares two NH bonds, and owns a positive charge that is distributed equally between both nitrogen atoms, thus representing a resonance structure. This new positive charge can generate new molecular forces with other molecules, causing a series of physiological phenomena (*i.e.* protein catalysis, protein stability, protein-protein interaction, and protein-ligand interaction) in the protein.

Human HSF1 have 13 histidine residues within three

important, preidentified functional domains. In the carboxyl-terminal domain (CTD) of hHSF1, five histidines were distributed in two largely unfolded regulation/trans-activation domains (RTD). An analysis of circular dichroism and size-exclusion HPLC proved that hHSF1CTD can form a more compact, partially-structured shape by exposure to an acute acidic pH (pH at 4), high temperatures, or cationic detergents.¹⁴ The multiple stress-induced structural folding or deletion of hHSF1CTD could result in its trimerization and DNA-binding, which suggests the dispensable role of pH-sensitive histidines in hHSF1CTD.

Another four histidines were observed in the hHSF1 trimerization domain (TD) which belongs to a triplestranded, heptadrepeated coiledcoil motif. Trimerization assays by sedimentation equilibrium and cross-linking chemical suggested that under alkaline condition (pH = 8.8 in reaction buffer), Kluyveromyces lactis HSF (KIHSF)TD can still form a homotrimer in vitro. This concurs with a similar study of hHSF1, which reported that these histidines are not essential in mild acidic-pH mediated HSF1 activation.¹⁵ Finally, this study focused on the last four residues (His45, His63, His83, and His101) located in the hHSF1 DNA-binding domain (DBD), because DBD is the most conserved and important functional region in HSF1. In this study, we examined whether their protonation states can induce hHSF1-DBD to interact with each other and achieve DNA-binding capability.

The histidine residues in the HSF1-DBD were replaced with alanine by PCR-mediated mutagenesis. The DNA binding activities of these histidine mutants of HSF1 which were induced by heat shock or low-pH (pH at 6.0), were then examined Figure 1(c)). After heat shock, the His63A mutant mutants of HSF1 exhibited no DNA-binding activities, whereas the other mutant showed heat-induced DNA-binding activities. Moreover in low pH environment, His63A and His83A lost their low-pH induced DNA-binding activities.

The His63 residue located in the ‘turn’ region of a helixturnhelix motif (the most highly conserved segment in HSF) was identified in all known HSFs. The crystal structure of the KIHSF-DBD (PDB id: 3HTS) complexed with the synthetic HSE clearly showed that the His242 residue, a homolog of His63 in hHSF1, contacts directly (1.9 Å) the phosphate backbone of the thymine base in the major groove

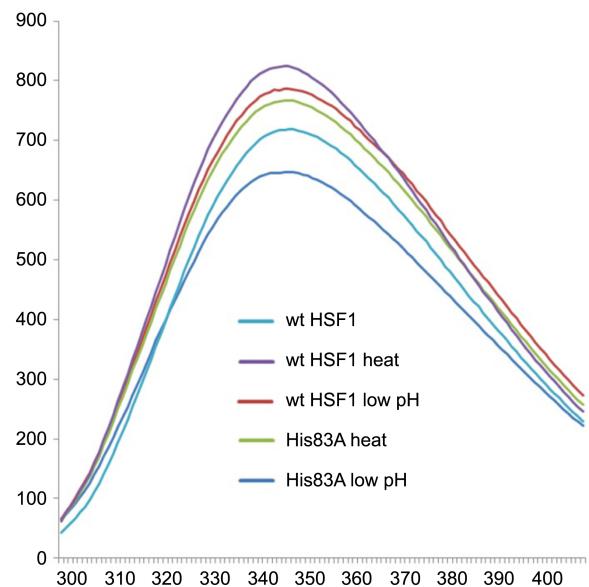


Figure 3. TFS in HSF1s under the heat, or low-pH conditions. The protocols were shown in the experimental section.

of HSE in the third α -helix of DBD (Figure 2, Green Color). The DBD of hHSF1 (PDB id: 2LDU) was aligned with the one from KIHSF. In human HSF1 (which possess intrinsic stress-sensing capability), this histidine homolog (His63) buries itself inside the DBDs (Figure 2, Red Color). Unlike the higher eukaryotic human HSFs, KIHSF is bound constitutively to HSE using this always-exposed residue, regardless of whether they are under non-stressful and stressful conditions. This suggests that in higher eukaryotic organisms, environmental stress can cause the exposure of this histidine out of the HSF-DBD surface for HSE binding. Overall, the His63 residue in hHSF1 is a key important residue for constitutive and heat-induced HSE-binding, but not for the acidic pH-induced HSE-binding.

The residue, His83, which exists in the loop region of hHSF1DBD, is conserved positionally in every stress-responsive HSF isoform (mouse, rat and human). The loop region in mouse HSF1 suppresses the formation of the HSF1 trimer under basal conditions and is essential for heat-inducible trimerization in a purified system *in vitro*. This suggests that this domain plays a critical role in the HSF1 heat-stress-sensing mechanism. In yeast *Saccharomyces cerevisiae*, the wing of HSF (the loop homolog in hHSF1) does not appear to contact DNA, but is involved in the protein-protein interactions, possibly within a trimer or between adjacent trimers.¹⁶ A mutation of His83 in hHSF1 inhibits low-pH induced DNA-binding *in-vitro* (Figure 1(c)). Therefore, Cluspro 2.0 (a web server for protein-protein docking) was used to simulate a set of hHSF1-DBD homotrimers.¹⁷ After screening the output data, one possible structure of the hHSF1-DBD homotrimer appears reasonable (Figure 2(b)). In this predicted structure, the loop regions and hydrophobic interfaces (containing four β -sheets) from the three hHSF1-DBDs cluster together to form a hydrophobic core, together with three exposed, same directional, DNA-

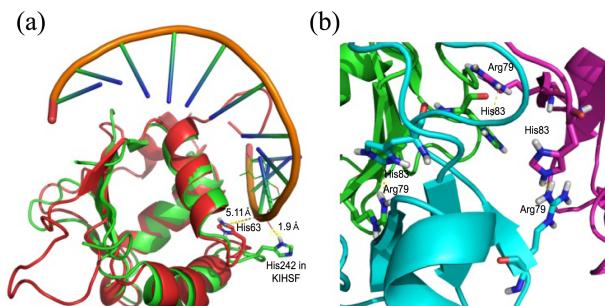


Figure 2. (a) Possible His63-participant intermolecular interaction in hHSF1-DBD with HSE (b) Cluspro 2.0 generated hHSF1-DNA trimer suggest a possible intermolecular interaction between Arg79 and His83.

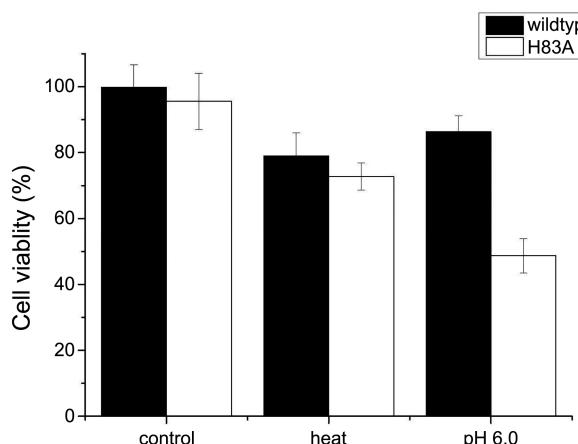


Figure 4. Cell viabilities were measured using the MTT assay at 24 hours after heat- (42°C for 1h) or pH(6.0)- induced cells. Cell lines were mouse embryonic fibroblasts $\text{hsf}1^{+/+}$ (wild-type) and $\text{hsf}1^{-/-}$ harboring the H83A mutant of HSF1. Each data point represents the mean and S.D.

recognition regions (α -helix 3). Protonated His83 could generate extra-molecular interacting forces (measured distances were 4.38 \AA , 4.11 \AA and 2.73 \AA) with Arginine 79 (Arg79) from another hHSF1-DBD (Figure 2(b)). A recent biophysical study reported that a protonated histidine side chain group tends to form enhanced like-charged contact pairs with arginine, when the distance is less than 6 \AA . Therefore, the acidic-pH-induced, intermolecular force between Arg79 and His83 might help the three hHSF1-DBDs achieve DNA-binding ability. Trp-fluorescence spectroscopy was performed to further confirm our hypothesis. By general excitation at 280 nm , most of the emission signals in the protein are due to excitation of the tryptophan residues.¹⁸

Human HSF1 has three tryptophan residues, two of which (Trp23 and Trp37) are located in the HSF1 DNA-binding domain (DBD). TFS has been adopted previously to prove that the Trp37 residue is involved in an intermolecular π - π stacking interaction that results in heat-induced HSF1 trimerization. In Figure 3, heat-induced (at the temperatures of 42°C) hHSF1-activation induced a stronger signal than that in the low-pH induced hHSF1-activation (Figure 3), suggesting a potential structural change (a more folding state) in HSF1. However, the His83A mutant HSF1 in a low-pH buffer exhibited a very weak signal compared with the samples after heat-induced.

To further identify the above interactions on the mammalian cell-line level, mouse HSF1 was cloned into the pCDNA3.1 vector, PCR-mediated point mutated, and transfected into HSF1-knock-out ($\text{hsf}1^{-/-}$) mouse embryonic fibroblasts. MEF $\text{hsf}1^{+/+}$ was used as the positive control (wild-type). The cell viability was determined by an MTT assay. As shown in Figure 4, the wildtype cells exhibited high resistance to heat- or pH-treated cell death. On the other hand, almost fifty-percent cell death was observed in the H83A cells treated with acid pH stress.

The expression of HSP70 in these cell lines were confirmed

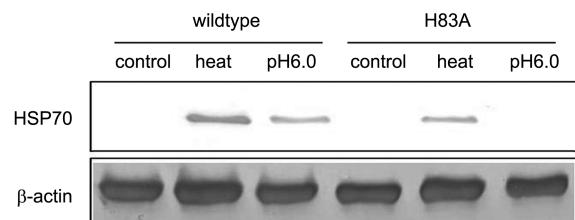


Figure 5. Expression analysis of HSP70 by western blot. The cell lysates were prepared and subjected to western blotting using HSP70 and anti-actin antibodies. Beta-actin serves as the protein loading control.

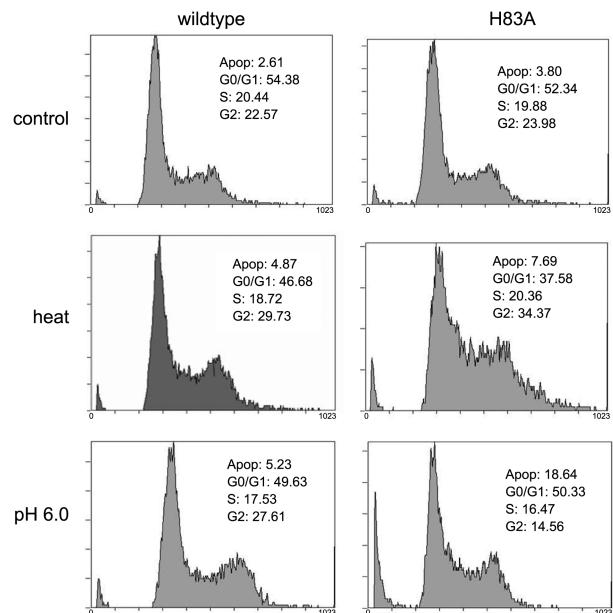


Figure 6. Cell cycle analysis. Cells were fixed and cell-cycle distribution was analysed by flow cytometry using a FACScan flow cytometer.

by western blotting. HSP70 can inhibit apoptosis of mouse embryonic fibroblasts, whereas the normal transcription of HSP70 requires the natural trimerization of HSF1. As a result, both the wild-type and H83A cells revealed HSP70 induction under heat stress. The expression of HSP70 was also observed in the pH-induced wild-type cells, but the amount was lower than that exposed to heat stress. No induction of HSP70 was observed in the $\text{hsf}1^{-/-}$ cells transfected with the H83A mutant (Figure 5).

Our previous study reported that the cells lacking a normal HSF1 failed to arrest in the G2 phase, resulting in cell apoptosis.¹⁰ The present study determined the cell cycle distribution in the cells. As shown in Figure 3(a), both the heat- and pH-induced $\text{hsf}1^{+/+}$ cells showed a significant increase in the percentage of the G2 phase. The heat-induced H83A cells showed similar G2 cell cycle arrest with that of the wild-type cells. Under acid pH stress, however, the H83A cells showed a high sub-G1 peak corresponding to an increased in the population of apoptotic cells to 18.64% from a control value of 3.80% (Figure 6). These results further proved the importance of the His83 residue in pH-dependent HSF1 activation.

Conclusion

Human HSF1 could be activated by the mild acidic environment (pH~6.0), which suggests the involvement of the imidazole group of histidine residues. Our results including the EMSA experiment, Trp-fluorescence spectroscopy, and protein structural analysis showed that the residue His83 is the key residues for pH-dependent hHSF1-activation. The experiments using the mouse embryonic fibroblasts highlight the importance of His83 in HSF1 activation or the HSF1-mediated transcription of heat shock proteins.

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